

# Acoustic Droplet Vaporization, Cavitation, and Therapeutic Properties of Copolymer-Stabilized Perfluorocarbon Nanoemulsions

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**Abstract.** Acoustic and therapeutic properties of Doxorubicin (DOX) and paclitaxel (PTX)-loaded perfluorocarbon nanoemulsions have been investigated in a mouse model of ovarian cancer. The nanoemulsions were stabilized by two biodegradable amphiphilic block copolymers that differed in the structure of the hydrophobic block. Acoustic droplet vaporization (ADV) and cavitation parameters were measured as a function of ultrasound frequency, pressure, duty cycles, and temperature. The optimal parameters that induced ADV and inertial cavitation of the formed microbubbles were used *in vivo* in the experiments on the ultrasound-mediated chemotherapy of ovarian cancer. A combination tumor treatment by intravenous injections of drug-loaded perfluoropentane nanoemulsions and tumor-directed 1-MHz ultrasound resulted in a dramatic decrease of ovarian or breast carcinoma tumor volume and sometimes complete tumor resolution. However, tumors often recurred three to six weeks after the treatment indicating that some cancer cells survived the treatment. The recurrent tumors proved more aggressive and resistant to the repeated therapy than initial tumors suggesting selection for the resistant cells during the first treatment.

**Keywords:** Cancer Therapy, Targeted Drug Delivery, Nanobubbles, Perfluoropentane, Ultrasound.

**PACS:** 87.50.yt, 87.63.dh.

## INTRODUCTION

During the last decade, a number of novel modalities for a targeted tumor therapy have been suggested. These modalities are based on developing stimuli-responsive nanoparticles that release their drug load in response to environmental or physical stimuli, such as pH, hyperthermia, light, or ultrasound. In our previous work, we have developed the nanoemulsions that converted into nano- and microbubbles *in situ* upon injection or under the action of therapeutic ultrasound [1], [2]. These systems combined passive tumor-targeting capacity with ultrasound responsiveness and could be used for combining ultrasonography with ultrasound-mediated chemotherapy. Here we describe acoustic properties of these systems.

The nanoparticles we have developed are composed of nano- or micro-scale echogenic emulsions that convert into nano- and/or microbubbles *in situ* upon injection and tumor sonication. A set of their properties includes drug carrying, tumor-

targeting, enhancing intracellular drug delivery, and enhancing the ultrasound contrast of the tumor. The cores of the nanoemulsion droplets or nanobubbles were formed by organic perfluoro- compounds; in this work, perfluoropentane (PFP) was used; the PFP was encased in the walls formed by the biodegradable amphiphilic block copolymer, poly(ethylene oxide)-co-poly(L-lactide) (PEG-PLLA) or poly(ethylene oxide)-co-polycaprolactone (PEG-PCL).

Due to the impedance difference from the surrounding medium, not only perfluorocarbon bubbles but also droplets have echogenic properties; however bubbles manifest higher echogenicity than droplets, which allows using ultrasound imaging to monitor acoustic droplet vaporization (i.e. the ADV effect that was discovered and investigated in a series of works by Kripfgans, Fowlkes *et al.* for a different type of perfluorocarbon emulsions [3], [4]). Besides producing high ultrasound contrast, bubbles serve as potent enhancers of ultrasound-mediated drug delivery. Therefore nanodroplet vaporization *in situ* to generate bubbles is highly desirable for both ultrasonography and drug delivery. The PFP has a boiling temperature of 29 °C thus producing nanoemulsions at room temperature. The ADV effects in PFP nanoemulsions stabilized by PEG-PLLA and PEG-PCL are described below. Another issue addressed in the paper is related to the ADV effect and bubble cavitation in highly viscous gel systems used as tissue models. This information is relevant to acoustic effects produced in tumor interstitium after bubble extravasation. The information on the acoustic properties of these novel formulations obtained in this study was used for optimizing *in vivo* tumor imaging and therapy.

## MATERIALS AND METHODS

*Block copolymers.* Block copolymers used in this study were bought from Polymer Source (Quebec, Canada). The PEG-PLLA copolymer had a total molecular weight of 9,700. The PEG-PCL copolymer had a total molecular weight of 4,600 D.

*Micellar solutions and drug loading.* Micellar solutions of the block copolymers were prepared by a solvent exchange technique as described in details previously [1]; DOX loading into the micelles was performed at the micelle preparation stage. Genexol-PM was bought from Samyang Corp. (Daejeon, South Korea) and dissolved in PEG-PLLA micellar solution.

*Preparation of nanoemulsions.* An aliquot of PFP was pipetted into a corresponding micellar solution and sonicated by 20-kHz ultrasound in ice-cold water.

*Nanoparticle size distribution.* Size distribution of nanoparticles was measured by dynamic light scattering at an angle of 165° using Delsa Nano S instrument (Beckman Coulter, Osaka, Japan) equipped with a 658 nm laser and a temperature controller. Size distribution was analyzed using Non-Negative Least Squares (NNLS) method.

*Sonication.* Unfocused 1- or 3-MHz ultrasound was generated by an Omnisound 3000 instrument (Accelerated Care Plus Inc, Sparks, NV) equipped with a 5 cm<sup>2</sup> transducer head.

*Cavitation activity.* Cavitation activity was assessed by measuring subharmonic, harmonic, and broadband noise amplitudes in a scattered beam. The microbubble formulation inserted in a Samco polyethylene transfer pipette (5 mm internal diameter, 0.3 mm wall thickness, Fisher Scientific, Pittsburg, PA) was positioned at a distance

of 0.5 cm from the transducer that was housed in an open glass tank containing filtered distilled degassed water. To minimize possible standing wave formation, a 2.5 cm thick rubber liner was mounted opposite the transducer. Ultrasound pressure was measured using a needle hydrophone (HNR-1000, Onda, Sunnyvale, CA) with a 20 dB preamplifier (AH-1100, Onda, Sunnyvale, CA). The hydrophone was placed perpendicular to the beam direction at a distance of 3 cm from the sample.

*Monitoring acoustic droplet vaporization by visual observation and ultrasound imaging.* Formation of the microbubbles from the nanodroplets under the action of ultrasound was monitored visually and by ultrasound imaging using a 7.5-MHz scanner (Scanner 250, Pie Medical, Maastricht, The Netherlands).

*Cells and tumor models.* Ovarian cancer A2780 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured and inoculated to nu/nu mice as described previously [1].

## RESULTS AND DISCUSSION

### Acoustic droplet vaporization

These experiments were performed for two types of the droplet stabilizing copolymers (PEG-PLLA and PEG-PCL) and two types of matrices (liquid (PBS) and gel (0.2% agarose or a bovine plasma clot) for 1-MHz or 3-MHz ultrasound. Some experiments were performed using low-frequency CW 90-kHz ultrasound generated in the ultrasound bath (SC-100, Sonicator Instrument Co., Copiague, NY) with intensity close to that of 1-MHz ultrasound (peak-to-peak pressure of 0.7 MPa). Ultrasound intensities that (a) induced the formation of the first visible bubbles; and (b) induced droplet-to-bubble transition in the whole volume of the sample were recorded.

For both emulsion types, the first visible bubbles were always formed on the distal wall of the container at low ultrasound intensities thus suggesting their nucleation in the surface crevices. In liquid systems, the onset of the droplet-to-bubble transition in the whole volume of the sample was accompanied by intensive bubble coalescence into the large bubbles that were raised to the sample surface thus mimicking sample boiling. In what follows, we call this phenomenon “global ADV”. The threshold of the global ADV was noticeably higher than that for the formation of first bubbles on the wall surface.

*Effect of the type of droplet-stabilizing copolymer.* For the droplets stabilized by a PEG-PCL copolymer, the ADV threshold was lower than that for the bubbles of the same composition stabilized by a PEG-PLLA copolymer; as an example, at room temperature and under 1-MHz ultrasound with a 20% duty cycle (1.2 ms pulse duration and 4.8 ms inter-pulse interval), the global ADV threshold for the 1% PFP/0.25% PEG-PCL system was 0.57 MPa compared to 0.85 MPa for the droplets of the same composition stabilized by PEG-PLLA.

*Effect of the duty cycle.* The ADV threshold strongly depended on the ultrasound duty cycle and was significantly higher for pulsed ultrasound as compared to CW ultrasound. In PFP/PEG-PCL system, five-fold higher nominal ultrasound energy was required for initiating the global ADV effect by 1-MHz ultrasound of 20% duty cycle

than by CW ultrasound; moreover, in PFP/PEG-PLLA systems, pulsed ultrasound with 20% duty cycle did not induce global ADV effect while CW ultrasound did.

*Effect of temperature.* For both types of the emulsions, the ADV threshold was lower at 37 °C compared to room temperature. As an example, for 1% PFP/0.25% PEG-PCL system sonicated for 1 min by 1-MHz ultrasound with 20% duty cycle, ADV threshold dropped from 0.68 MPa at room temperature to 0.44 MPa at 37 °C.

*Effect of ultrasound frequency.* For both types of the emulsions, the ADV threshold was higher for 3-MHz compared to 1-MHz ultrasound.

*Effect of gel matrices.* While liquid systems can mimic bubble behavior in blood vessels, much more viscous environment surrounds the bubbles extravasated into the extracellular matrix in tumor tissue. Introduction of the nanodroplets into a gel matrix substantially hampered global formation of large bubbles. Visual observations and ultrasound imaging indicated the formation of isolated large bubbles (FIGURE 1(a), sonication by CW 1-MHz ultrasound at 1.18 MPa peak-to-peak pressure for 1 minute); for comparison, in PBS suspensions, an intensive global “boiling” was observed almost immediately at a pressure as low as 0.3 MPa. However the absence of the global formation of large bubbles does not signify the absence of the droplet-to-bubble transition. As indicated by the generation of harmonics and broadband noise (see below), ultrasound did induce droplet-to-bubble transition in gel matrices; however, gel precluded intensive bubble coalescence in the sample volume. FIGURE 1(a) suggests that the isolated bubbles formed under ultrasound initiated droplet-to-bubble transition in their immediate vicinity and grew by coalescence with these bubbles. This effect was much stronger at lower ultrasound frequencies; an example for gel sonication by 90-kHz ultrasound at 0.7 MPa is shown in FIGURE 1(b). For the optimal intratumoral drug delivery and imaging, it appears reasonable to modulate megahertz frequency range (which is required for ultrasound focusing) by a hundred kilohertz frequency range in therapeutic ultrasound.

## Bubble Cavitation

For the effective drug delivery, the formation and cavitation of the nano/microbubbles from the nanoemulsions are extremely beneficial because the oscillation and cavitation of the bubbles trigger the release of the encapsulated drug and also perturb cell membranes thus enhancing the intracellular drug uptake. In the present study, the cavitation effects have been explored for unfocused ultrasound at a frequency of 1 MHz. The appearance and amplitudes of harmonic frequencies and broad band noise were monitored in Fast Fourier Transform emission spectra. The representative data on the relative subharmonic peak amplitudes are shown in FIGURE 2(a) for the PFP/PEG-PCL emulsion in PBS and in FIGURE 2(b) for the droplets inserted in the agarose gel. The threshold of the stable bubble cavitation (about 0.5 MPa) is clearly seen in FIGURE 2(a) (but not in FIGURE 2(b) because the gel comprised some preexisting bubbles). To characterize inertial cavitation, we measured mean relative amplitude of a broadband noise in the frequency intervals that avoided fundamental, harmonic, and superharmonic frequencies. The data obtained clearly indicated that inertial cavitation of the bubbles proceeded in both liquid and gel systems at the ultrasound pressure of 1.18 MPa that was chosen for *in vivo*

experiments. The promising results of *in vivo* studies are shown in FIGURE 3. Ovarian carcinoma tumors were grown in the left and right flank of a mouse; PTX-loaded PEG-PLLA nanoemulsions were injected systemically; only one (the right) tumor was sonicated. The unsonicated tumor grew with the same rate as control indicating a strong retention of the drug in the bubbles while the sonicated tumor was resolved after four treatments.

Very promising chemotherapy results were also obtained with DOX-loaded PEG-PCL nanoemulsion

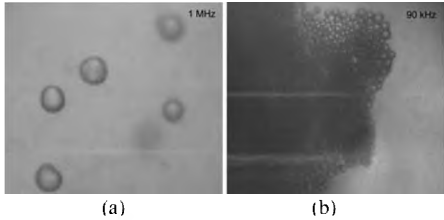


Figure 1. PFP/PEG-PCL microbubbles formed from the nanodroplets inserted in the bovine plasma clots under ultrasound of 1 MHz (a) or 90 kHz (b).

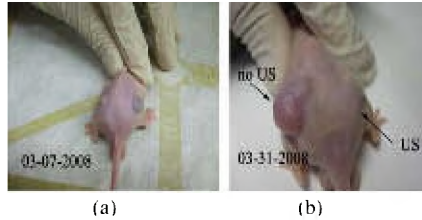


Figure 3. Treatment of the ovarian carcinoma by PEG-PLLA nanobubble-encapsulated paclitaxel; (a) – before treatment; (b) – three weeks after treatment.

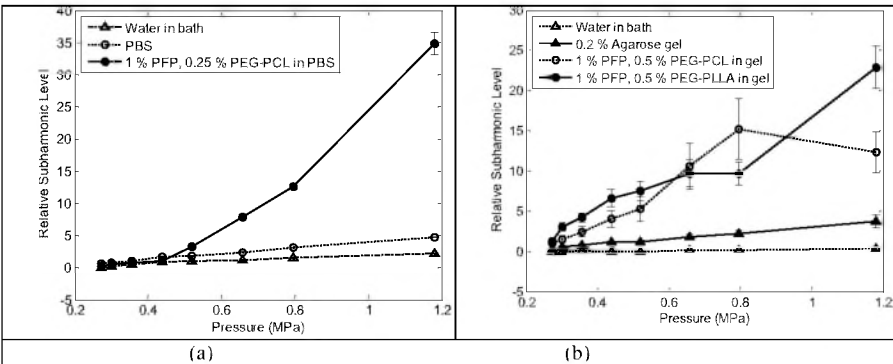


Figure 2. Stable cavitation as characterized by relative subharmonic amplitudes of the bubbles formed by the ADV from the droplets inserted in PBS (a) or agarose gel (b)

## ACKNOWLEDGMENTS

This work was supported by the NIH R01 EB1033 Grant to N. Rapoport.

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